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Abscisic Acid and Tissue Desiccation Alters Ribonuclease I Activity in Winter Barley without Cold Acclimation

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ABSCISIC ACID AND TISSUE DESICCATION ALTERS RIBONUCLEASE I ACTIVITY IN WINTER BARLEY WITHOUT COLD ACCLIMATION

BY

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ABSCISIC ACID AND TISSUE DESICCATION ALTERS
RIBONUCLEASE I ACTIVITY IN WINTER BARLEY
WITHOUT COLD ACCLIMATION

This thesis is approved as a creditable and independent investigation by a candidate of the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

Ribonuclease (RNase) has been used to study phenotypic differences between freeze-resistant and susceptible cultivars of winter barley. Kenefick et al. (26) and Johnson et al. (23) have demonstrated that RNase I activity is greater in freeze susceptible than in freeze resistant cultivars. Hawthorne et al. (21) used rocket immunoelectrophoresis to quantitate RNase levels of 4-day seedlings from a freeze resistant and susceptible cultivar. He reported that both cultivars of barley contained equal amounts of RNase. However, when RNase from each cultivar was assayed, enzyme from the susceptible cultivar showed a higher hydrolysis rate compared to RNase from the resistant cultivar, indicating that the difference in activity was a catalytic property of the enzyme.

Initial procedures for the extraction of RNase from 4-day barley seedlings in this laboratory used 0.4 M KCl (27). Wong (50) also used this salt to extract RNase from the same tissue and was able to isolate two peaks of RNase activity by gel filtration. He showed the earliest eluting RNase peak to contain less enzyme than the later emerging major peak (RNase I). Hawthorne (21) developed antibody to RNase I, using the extraction procedure described by Wong (50), and showed it to be cross-reactive with the first eluting RNase peak (unpublished results). Bunkers (6) changed to $(NH_4)_2SO_4$ precipitation in purification for improved recovery of the enzyme and subsequently found that 0.6 M

$(NH_4)_2SO_4$ in the extraction instead of 0.4 M KCl also increased yield of both RNase I and II. He also reported that addition of Triton X-100 in the column chromatography steps after tissue extraction was needed to maintain RNase recovery, suggesting the presence of hydrophobic sites on the enzyme. Bunkers subsequently showed the first eluting RNase peak from gel filtration to be a glycoprotein based on its affinity for Conconavalin-A Sepharose 4B (Con-A). The Con-A bound RNase had a pH optimum of 6.2, consistent with characteristics of RNase II (48). By contrast, the slower eluting peak in gel filtration did not bind to Con-A and had a pH optimum of 5.5 which is reported for RNase I (48). RNase II also showed characteristics of a glycoprotein in that it had a broad banding pattern on SDS-PAGE gels with a molecular weight in the range of 36,000 to 40,000, as compared to RNase I which showed a faster migration rate (MW 20,000 to 22,000).

Pietrzak et al. (34) used the Ouctherlony method to show cross-reactivity of antiRNase II with both RNase I and II purified from barley seed. This information indicates that RNase I and II have a common determinant site, suggesting a similar amino acid backbone and genetic origin. Baynes and Wold (3) have shown RNase A and RNase B to be interchangeable in the blood, suggesting that RNase in animals is also derived from a common metabolic pool.

The glycosylated (RNase II) and non-glycosylated (RNase I) forms of RNase appear to be programmed for specific locations within cells. Wilson (48,49) reported that RNase II, isolated from Zea mays, is bound in the microsomal fraction. Nishimura and Beevers (32) reported

soluble RNase to exist in isolated vacuoles of castor bean endosperm. Butcher et al. (7) describe mature vacuoles isolated from protoplasts of Hippeastium flower petals that contained a RNase which had a pH optimum characteristic of RNase I. Lusher and Mantle (29) have also identified RNase as a soluble acid hydrolase in vacuoles of Acetobularia.

Chrispeels and Varner (11) demonstrated that incubating barley aleurone layers in gibberellic acid (GA_3) resulted in the secretion of RNase, implicating it as a secretory protein. Ho and Varner (22) reported that abscisic acid (ABA) inhibited the GA_3-enhanced synthesis and secretion of RNase, with simular effects on alpha-amylase in barley aleurone layers. Thus, if RNase I and II do originate from the same genetic code, then evidence suggests that these enzymes are synthesized, processed, transported and secreted via the endomembrane system. Harris (19) indicates the major components of the endomembrane system of higher plant cells to include the endoplasmic reticulum (ER), the Golgi apparatus (GA), and their transition elements.

Increases in RNase activity have been associated with disease (8), drought (2), light (1), hormone regulation (11,13,31,37,46), mechanical damage (35), and senescence (4,40,43). Generally, these reports have not distinguished wheather changes in RNase activity are due to synthesis of new enzyme or alteration of a precursor protein. Pitt (35) concluded that increase in lysosomal RNase activity, due to mechanically damaged potato tubers, resulted from activation of pre-formed enzyme.

Schekman (41) reviewed the characteristics of two general groups of temperature sensitive pleiotropic secretory mutants in yeast which have also been shown to contain compartmented and pre-formed inactive secretory proteins. Class A sec mutants accumulate molecular intermediates of secretion at different sites along the endomembrane system. Whereas Class B sec mutants have been shown to accumulate enzymatically inactive forms of enzyme in the endoplasmic reticulum. These yeast data show a parallel with observations on RNase in barley tissue, in that the original procedures for extracting RNase in this laboratory with 0.4 M KCl may have not completely extracted RNase, as implicated by Bunkers' data (6). Thus, compartmentization of the enzyme, such as that seen for sec A mutants, is one explanation for differences in enzyme recovery between winter barley cultivars.

This research was initiated to obtain additional information about the causes for phenotypic variation of RNase I activity within winter barley cultivars. Unpublished data from this laboratory shows that antibody produced from RNase I obtained from two cultivars was mutually cross-reactive with antigen from the two cultivars of winter barley. The difference seen in RNase I activity between cultivars which can not be explained by the amount of RNase present may have simularities to that of inactive or incomplete forms of certain enzymes found in sec B mutants. Ferro-Novick et al. (15) reported such sec B mutants as having early inactive forms of invertase, carboxypeptidase, and alpha-factor that accumulated due to incomplete penetration into the ER lumen. They also reported that these enzymes were not extractable by Triton X-100.

Since antiRNase I was also cross-reactive with RNase II (34) and was found to be a glycoprotein (6), it is proposed that RNase I is post-translationally modified to form RNase II; a process completed in the endomembrane system (16). It is also proposed that export of RNase occurs via the endoplasmic reticulum-Golgi complex. Based on these assumptions it was of interest to determine if applications of GA_3 would cause differential secretion of RNase I or II from resistant and susceptible tissue, or somehow reflect different export programs, accounting for variation in enzyme content between the cultivars. The objectives of this study were:

Objective 1: To evaluate the effect of exogenous GA_3 on RNase in leaf tissue and the forms of RNase secreted from this tissue.

Objective 2: To compare tissue levels and secreted amounts of RNase between a freeze-resistant and susceptible cultivar in response to an antagonist of endogenous GA_3.

Objective 3: To evaluate the effects of tissue desiccation on tissue ABA accumulation and RNase levels between a resistant and susceptible cultivar.

Objective 4: To determine if alteration in RNase activity, resulting from changes in tissue ABA, would influence polynucleotide specificity.

MATERIALS AND METHODS

Plant Material: Seeds of Hordeum vulgare L. cv. `Herb´ (freeze resistant) and `Winter Malt´ (freeze susceptible) were germinated on blotting paper saturated with 0.1 mM $CaCl_2$ solution. Seedlings were grown for 6-days in the dark at 25°C. Shoots of 6-day seedlings were excised at the point of emergence from the seed. The coleoptile and leaf were then separated by gently pulling the exposed primary leaf from the surrounding coleoptile tissue. When cold acclimated tissue was used it was grown for 5 days in the dark at 25°C and then transfered to the acclimation chamber for 4 weeks at 2-4°C.

Sterilization and Incubation: All glassware and glass-distilled water was autoclaved. Incubating solutions used to float leaf tissue were sterilized by suction through a Millipore filtering apparatus containing a 13.8 cm^2 filter (0.45 micron pore size). These sterilized solutions and glassware were stored under a UV lamp in a vacuum hood in preparation for each experiment.

Tissue samples were surface sterilized by washing 3 min in 70% ethanol, 10 min in 10% commercial bleach, and rinsing 3 times with sterile water. Individual 2-gram samples of either leaf or coleoptile tissue were transferred to petri dishes in a vacuum hood. These samples were incubated from 6 to 36 hrs with one of the following solutions: (A) 20 mM 2(N-morpholino)ethanesulfonic acid (MES) buffer pH 5.5, (B) 0.1 mM ABA in solution A, (C) 0.1 mM dinitrophenol (DNP) in solution B, or (D) 0.1 or 1 mM GA_3 in either solution A or B. All

tissue samples were incubated in a total volume of 20 ml.

Duplicate tissue samples were used for studying the effect of light on ABA responses. Comparisons were made between tissue incubated under laboratory light vs. dark conditions. All incubated samples in this study were placed on a rotating shaker (60 cycles min^{-1}). After the designated incubation period, tissue samples were blot-dried with Kimwipes and immediately frozen. Incubated samples were individually wrapped in a double layer of aluminum foil and frozen in crushed dry ice for 15 min.

Tissue Extraction: The frozen samples were pulverized with a cold mortar and pestle and transfered to 30 ml test tubes maintained in crushed ice. All samples were warmed to 0°C before continuing with the extraction. Each sample was further homogenized by suspending the tissue with a Teflon pestle in 9 ml of prechilled extraction buffer (50 mM MES, pH 6.5) containing 600 mM ammonium sulfate, 250 mM sucrose, 20 mM magnesium acetate, and 5 mM 2-mercaptoethanol. The suspended samples were then filtered through a single layer of Miracloth fitted to a 48-mm-diameter Buchner funnel. Individual tissue extracts were centrifuged at 144,000g (0°C) for 2.5 hr in a model 40 rotor using a Spinco L-2 ultracentrifuge. After centrifugation, the soluble supernatant was further processed for RNase isolation.

Column Chromatography: Each sample was desalted using Bio-Rad P6-DG in a 1 x 55 cm column. All desalting columns were equilibrated with 50 mM Tris/HCl (pH 7.4), and 0.01% Triton X-100. Protein was eluted with the same buffer and protein peaks were detected by A_{280} absorbance

using a Beckman DU-50 spectrophotometer.

The bed volume for the Con-A step was 1.0 x 1.5 cm. Each column was equilibrated with Con-A buffer (50 mM Tris/HCl, pH 7.4), containing 0.01% Triton X-100 and 0.1 mM each of $MgCl_2$, $MnCl_2$, and $CaCl_2$. Samples were applied to the Con-A columns and the unbound RNase fractions were eluted by washing the column with Con-A equilibration buffer. The bound RNase fractions were eluted by using 500 mM alpha-methyl-D-mannopyranoside (Sigma). All of the above procedures were performed at 2°C.

RNase in the incubation media was first concentrated by ion-exchange chromatography with DEAE-Sepharose before being passed through Con-A. The bed volumes for DEAE were 1 x 2 cm. The DEAE columns were equilibrated with 50 mM Tris/HCl (pH 7.4), containing 0.01% Triton X-100. A 2-ml sample was applied to each column and was washed with 50 ml of equilibration buffer. The protein fraction containing RNase was eluted with 500 mM KCl.

Ribonuclease Assays: RNase activity was determined by the Tuve and Anfinsen procedure (45) with the following modifications. The assay solution consisted of 50 mM potassium acetate (pH 5.5), 160 mM KCl, 4 mM EDTA, 4 mg of yeast RNA (Holly preparation, Sigma), and 0.2 ml of the extracted protein sample. The final volume of the assay solution was 2.5 ml. Assays solutions were incubated at 37°C for either 30 or 60 min. After the incubation period, the reaction was cooled to 0°C before stopping the assay with 0.5 ml of 25% perchloric acid containing 0.75% uranyl acetate. After centrifugation at 2,200g for 10 min at

0°C, the supernatant was diluted 20-fold with double-glass-distilled water and the acid-soluble nucleotides were measured at 260 nm.

Calculations for the units of activity/mg protein have been described by Wilson (48), where a standard unit of enzyme is defined as giving an A_{260} of 1.0 min^{-1} mg $protein^{-1}$ for the acid-soluble nucleotides released. Bunkers (6) modified this calculation to account for a total assay volume of 2.5 ml + 0.5 ml stop reagent. For purposes of this report "relative activity" will be used to compare RNase activity between samples, where results of assays were standardized and expressed on a mg protein basis.

The polynucleotide assays for RNase activity were the same as described above, except that 0.5 ml (0.18 to 0.28 mg) of soluble protien was used in the reaction. The substrate consisted of 2 mg of either 5′-polyadenylic acid (Poly-A), 5′-polycytidylic acid (Poly-C), 5′-polyguanylic acid (Poly-G), or 5′-polyuridylic acid (Poly-U), all obtained from Sigma in the potassium salt form.

Units of activity for the reaction with Poly-A and Poly-U were calculated from A_{260} values, whereas the units of activity for the reaction with Poly-C and Poly-G were calculated from A_{280} and A_{250}, respectively (34). Total protein values were determined by the Bradford (5) method. Bovine serum albumin was used as the standard.

Abscisic Acid Extaction, Methylation and Determination: Six-day shoot tissue was excised and separated from the coleoptile as previously described. Two-gram samples of leaf tissue were allowed to desiccate under normal laboratory conditions for 3 hrs. The leaf

samples were then placed in plastic bags and held an additional 3 hrs in the dark at 25°C. After the desired desiccation and holding period, the samples were frozen in dry ice for 15 min and lyophilized to dryness for dry weight determination.

ABA was extracted as described by Dorffling and Dietmar (14) with slight modifications. Dry leaf samples were pulverized in a mortar and pestle, transfered to 30 ml test tubes, and twice extracted with 20 ml of 80% methanol (Fisher Scientific) containing 20 mg/L of 2-butylated hydroxytoluene (Koppers Chemical Co.) as an antioxidant. Samples were intermitently homogenized over a 30-min extraction period, using a Teflon pestle. Extracts were filtered through a Whatman#1 filter paper in a 48-mm-Buchner funnel. The samples were evaporated in vacuo at 35°C and then adjusted to pH 9.0 with 10 ml of 1% $NaHCO_3$. The samples were washed 3 times with anhydrous diethyl ether (Curtin Matheson Inc) using a separatory funnel. The diethyl ether washes were discarded and the water-soluble fraction, which contained the ABA, was filtered through Whatman#1 filter paper to remove pigments. Samples were then washed 3 times by phase separation, using ethyl acetate (Fisher Scientific). ABA was extracted from the water soluble fraction by lowering the pH to 3.0 and washing 3 times with ethyl acetate, each time the ABA partitioned into the organic phase. Lowering the pH protonates the ABA and thus allows it to fractionate into the nonpolar ethyl acetate. The ethyl acetate fractions were pooled and washed once with double glass-distilled water to remove any remaining organic mineral acids which could interfere with the derivatization process.

ABA samples were reduced to 1 ml under N_2 at 50°C. The samples were then methylated by reacting them 15 minutes with etherial diazomethane (42). Diazomethane was synthesized from DIAZALD (Aldrich), which was a generous gift of Dr. D. Matthees, South Dakota State University. Ether was removed from the samples under N_2 at 50°C and the remaining sample was diluted to a final volume of 10 ml with n-hexane (Fisher Scientific). All solvents used in this procedure were HPLC grade. Both ABA extraction and methylation were carried out in the darkness or under dim light to reduce isomerization of ABA due to UV light.

ABA was quantitated by GLC, using a Varian 3700 gas chromatograph equipped with a [^{63}Ni] ECD. A 6' x 4 mm ID column containing 3% Sp-2100: on 100/120 mesh supelcoport (Supelco Inc) with N_2 as the gas carrier. Column conditions were: oven temp 200°C, injector temp 230°C, ECD temp 350°C. Peak areas were obtained by injecting a 3 microliter sample of the methylated extract into the gas chromatograph. The standard recovery of ABA (Sigma) from tissue samples spiked with a standard ABA amount was 70%. ABA data in this report was adjusted for this loss.

Statistical Evaluation: In this report "significant" will be used only to refer to data which was shown to be statistically significant at $LSD_{0.05}$.

RESULTS AND DISCUSSION

RNase Activity in Response to GA3 and ABA: Preliminary experiments to alter tissue RNase or cause secretion of the enzyme from leaf tissue of the freeze-resistant cultivar incubated in GA_3 were unsuccessful. Since Ho and Varner (22) reported abscisic acid (ABA) to be an antagonist of GA_3 in the barley aleurone layer, it was also used as an antagonist in several of these preliminary experiments. Those tissue samples which were incubated in both GA_3 and ABA, or in ABA alone, showed a significant increase in tissue RNase activity (Fig 1) as compared to those incubated without ABA. Tissue floated in GA_3 showed no difference in the secretion of RNase into the incubation media compared to tissue incubated 20 hrs in MES buffer (0.11 vs 0.10 units of activity, respectively). There was also no difference between amounts of RNase (determined by activity measurements) secreted from tissue floated on ABA, or ABA plus GA_3 for 20 hrs (both showing 0.14 units of activity). However, there was a significant difference seen between the secretion of RNase from tissue incubated in ABA as compared to tissue incubated without ABA (0.14 vs 0.10 units of activity, respectively).

These results appear to indicate that ABA, and not GA_3, is the agent which stimulated alterations in RNase activity from leaf tissue. Poulson and Beevers (36) and De Leo and Sacher (13) also reported endogenous alterations in RNase activity due to incubation of excised leaf tissue in ABA. Thus, it appears that cells involved in seed

storage and germination, such as the barley aleurone layer, are programmed to stimulate RNase activity and secretion by GA_3, whereas excised leaf tissue was stimulated by ABA to alter RNase activity and secretion. Increases in RNase activity within leaf tissue, due to incubation with ABA, were associated with the ability to secrete RNase from the tissue. However, RNase activity in the incubation media may not be a true representation of the level of RNase secreted over a 20-hr period, since a time-decay loss in this enzyme may have occurred. Increases in RNase activity within the tissue, and especially the secretion of this enzyme, indicate that ABA is either directly or indirectly affecting the endomembrane system.

Affects of ABA Concentration on RNase Activity: Figure 2 shows that over a 28-hr test period the maximum increase in RNase activity occurred within leaf tissue of the resistant cultivar when incubated in 10^{-4} M ABA. The results show a significant increase of 33% in leaf RNase activity compared to tissue incubated 28 hrs without ABA. Accompanying this increase of RNase activity in tissue over the time-course was secretion of enzyme into the incubation media, which was positively related to ABA concentration (data not shown). There was also a concomitant increase in secreted protein with progressively higher ABA levels (Fig 2), which tends to imply a direct affect of ABA on membrane stability but without effect on the release of RNase. The results show evidence for either synthesis of new enzyme, activation of pre-formed enzyme, or selective retention of RNase activity.

ABA Incubation Time vs RNase Activity: When incubated in 10^{-4} M

ABA, leaf tissue from the resistant cultivar showed a significant increases in RNase activity for all test intervals over a 36-hr period as compared to leaf tissue incubated 28 hrs without ABA (Table 1). The maximum increase in RNase activity in leaf tissue occurred after 20 hrs, which represented a 40% increase in activity over that of tissue incubated without ABA.

RNase activity was also measured in the incubation media over a 36-hr period (Fig 3). Increases in secreted RNase appear to be positively associated with increases in RNase activity within leaf tissue over the first 20 hrs of incubation with ABA. However, after the first 20 hrs of incubation the secreted level of RNase remained constant for the next 12 hrs. Increases in RNase activity, along with concomitant increases in secretion of RNase such as those reported here, may indicate synthesis of new enzyme. Senescing morning glory flowers incubated in D_2O incorporate the isotope into RNase (4) indicating synthesis of new enzyme. Increases in RNase from senescing Rheoe leaf sections have been reported to be blocked by inhibitors of RNA and protein synthesis (40). Since senescence of tissue is generally associated with elevated levels of endogenous ABA (10), it is apparent that de novo synthesis of new enzyme could account for increased activity in situ and also for the secretion from barley leaf tissue.

In contrast to leaf samples, excised coleoptile tissue showed no difference in RNase activity over a 36-hr incubation period with 10^{-4} M ABA (Table 1). However, the coleoptile samples which were incubated in ABA showed an increase in protein content without an associated export

into the medium. Coleoptile tissue incubated 12 hrs in ABA resulted in the most significant increase in protein compared to coleoptile incubated 28 hrs without ABA (0.37 vs 0.44 milligrams of protein/ ml). In this laboratory coleoptile has been shown to stop growth 4 days after germination (unpublished data). The information reported here appears to indicate that ABA stimulates protein synthesis in 6-day coleoptile tissue.

Type of RNase Activity Stimulated by ABA: Based on Bunkers information (6), that RNase II can be separated from RNase I using Con-A, the purpose of this test was to determine which form of RNase accumulated within tissue, and which was secreted from tissue into the incubation medium as a result of ABA treatment. The results clearly indicate that increased RNase activity within the tissue (Table 2), and that secreted from the tissue (Fig 3), were due only to shifts in RNase I.

Table 2 shows that coleoptile contains approximately equivalent amounts of RNase I and II. If ABA caused increased permiability to membranes one might expect to see leakage of both RNase I and II from the coleoptile tissue. However, there was no detectable increase in RNase activity found in the coleoptile bathing media which was concentrated on DEAE and passed through Con-A (data not shown). Because secretion was not observed from coleoptile tissue it was not considered for further experiments.

Affects of DNP and Withdrawal of ABA on RNase I: It appeared that RNase I was either synthesized or that there was activation of pre-

formed enzyme due to treatment with ABA. It was assumed that synthesis of protein requires an energy source such as ATP. Since DNP is a known uncoupler of oxidative phosphorylation (18), it was used to compare alterations in secretion as well as alteration to RNase activity within leaf tissue of the resistant cultivar.

Figure 4 shows that all leaf samples incubated with ABA and DNP contained significantly less RNase activity than did tissue incubated 20 hrs in ABA alone. The loss in RNase activity from tissue incubated in ABA and DNP appears to be inversely related to an export of RNase into the incubation medium (Table 3). Tissue treated 20 hrs in ABA and DNP exported significantly greater amounts of RNase than did tissue incubated 20 hrs in ABA alone (0.41 vs 0.25 units of activity, respectively). Due to the increased RNase activity in the bathing medium, along with the paralleled loss of activity in the tissue when incubated with DNP, it appears that DNP may causes an overall degradative affect on the plasma membrane which allows for increased export of RNase from the tissue.

An additional variation in this study was to determine if RNase I would continue to increase in the tissue, or be secreted, if ABA was removed from the incubation media after an induction period. Tissue incubated 6 hrs in ABA, followed by 14 hrs in MES buffer, resulted in a significant increase in RNase activity compared to tissue incubated 20 hrs in ABA alone (Fig 4). This procedure also resulted in a significant decrease in RNase secreted from tissue when ABA was removed, compared to a treatment where tissue remained 20 hrs in ABA

(0.07 vs 0.25 units of activity, respectively). These results indicate that an initial stimulation by ABA caused an increase of RNase I activity, and removal of ABA does not irreversibly alter this activation process. The continued presence of exogenously applied ABA may have a pronounced effect on the plasma membrane which allows for either an increased leakage or active transport of protein. Stillwell and Hester (44) reported that ABA can increase membrane permiability by interacting with phosphatidylethanolamine.

From the data reported here it is evident that ABA alone does not cause the same effects seen with DNP addition. Mansfield (30) reported that ABA itself serves as an uncoupler of oxidative phosphorylation. However, DNP caused a decrease in RNase activity in tissue, even in the presence of ABA, indicating either an activation of RNase I in tissue or that synthesis of new enzyme occurred. A supply of metabolic energy appears necessary for the changes observed. Varner and Mense (47) showed that DNP inhibited both synthesis and secretion of alpha-amylase in barley aleurone layers and claimed that expenditure of oxidative phosphorylation energy is needed to move alpha-amylase to the plasma membrane.

Since tissue samples which were incubated with DNP showed RNase activity levels significantly lower than tissue incubated without ABA or DNP, it appeared that removing the source of respiratory energy may have also caused a loss of membrane function, allowing for leakage of cell contents. This was evident when comparing tissue protein levels where DNP appears to cause a greater loss. Tissue samples which were

incubated in DNP and ABA contained approximately 50% of the protein resulting in tissue incubated in ABA alone (data not shown). The same was true for tissue incubated 6 hrs with ABA and DNP followed by 14 hrs in buffer, compared to tissue incubated 6 hrs in ABA followed by 14 hrs in MES buffer (.37 vs 1.0 milligram protein/ ml, respectively). The effect of DNP physically altering membranes cannot be disregarded in contrast to its role of uncoupling ATP needed to maintain biological function of membranes.

It was also of interest to determine if removing the energy source produced by light would cause an effect on the secretion and alteration of RNase I activity in leaf tissue. Leaf tissue incubated with ABA for 20 hrs was yellowish in color and rolled, appearing no different from that of non-treated 6-day tissue grown in the dark. Whereas tissue incubated in buffer alone became green in color and remained flat under laboratory light conditions. Similar results were reported by Poulson and Beevers (37) for etiolated barley leaf segments. Even though these results appear to show that ABA affects the chloroplasts by preventing the greening of the tissue, no difference was observed between the freeze-resistant and susceptible cultivar in the secretion or alteration of RNase I activity due to incubation in light (data not shown). These results suggest that light did not further enhance RNase I activity in barley leaf tissue caused by ABA. These results also indicate that incubation of tissue in light did not influence the transport of RNase I from excised barley leaf tissue floated on ABA.

ABA affects on Freeze-Resistant vs Susceptible Tissue: The purpose

of this test was to determine if ABA would differentially stimulate secretion of RNase from the freeze-resistant cultivar compared to the susceptible one. RNase I activity from the resistant tissue showed a significant increase of 29% when floated on ABA compared to tissue floated on buffer alone (Fig 5). By contrast, similar treatment of the susceptible cultivar resulted in no difference in RNase activity within the tissue as compared to tissue incubated without ABA. However, the susceptible cultivar did show a significant increase in the secretion of RNase into the incubation media when incubated with ABA (Table 4), as compared to tissue incubated without ABA (0.15 vs 0.08 units of activity, respectively). By contrast, there was no significant difference in RNase activity from the incubation medium bathing the leaf tissue from the resistant cultivar with and without ABA, 0.15 units and 0.12 units, respectively. These results showed that ABA stimulated the secretion of RNase I from both cultivars of tissue. The susceptible cultivar did not accumulate RNase I activity to the same level as the resistant tissue (Fig 5), yet exported the enzyme to the same degree when stimulated by ABA and based on activity comparisions, suggesting that the two cultivars may differ in their export mechanisms.

RNase Activity After Drought Stress: The possibility existed that exogenous ABA may influence the integrity of the plasma membrane thereby causing leakage of RNase as opposed to an active transport. It was therefore desirable to seek a means for altering ABA within the tissue without direct application of ABA. Creelman and Zeevart (12) reported that desiccation of excised leaf tissue from Spinacia

aleracealas resulted in an increase of endogenous ABA levels by as much as 10-fold. The purpose of this test was to determine if desiccation of excised barley leaves would alter endogenous ABA levels and therefore affect RNase I activity.

When leaf tissue from the resistant and susceptible cultivar was desiccated for one hour an approximate 2-fold increase in endogenous ABA was observed in tissue from both tissue sources (Fig 6). However, after 3 hrs of desiccation, a significant difference between the two cultivars was seen. The resistant cultivar showed a 6 to 7-fold increase in ABA, whereas the susceptible cultivar showed a 10 to 11-fold increase in ABA when compared to non-desiccated tissue.

The resistant cultivar showed a slower loss of water from the tissue than did the susceptible cultivar (Fig 7). Since ABA is known to close the stomata and reduce respiration (38), it is interesting that the susceptible cultivar, which contained a higher concentration of ABA, showed a faster rate of water loss.

Tissue from the resistant cultivar which was desiccated for 6 hrs showed RNase activity equivalent to tissue only incubated 20 hrs in ABA (Fig 5). Desiccated tissue subsequently incubated 14 hrs in MES buffer showed an additional increase in RNase activity within the tissue as compared to desiccated tissue without incubation (2.8 vs 2.6 units of activity, respectively). Interestingly, susceptible tissue which was desiccated 6 hrs resulted in a significant increase of RNase activity within the tissue (Fig 5) as compared to tissues only incubated 20 hrs in ABA (3.0 vs 1.9 units of activity, respectively). In contrast to

the desiccated tissue from the resistant cultivar, susceptible tissue which was desiccated 6 hrs, and then incubated 14 hrs in MES buffer, showed a significant loss of 13% RNase activity within the tissue as compared to desiccated tissue without incubation.

This data indicates that resistant and susceptible tissue have different responses to endogenous ABA as compared to exogenous ABA (Fig 5 and 7). Additionally, the susceptible tissue which was desiccated and then incubated in buffer showed twice the secretion of RNase as did the resistant tissue which recieved the same treatment (0.14 vs 0.07 units of activity, respectively, Table 4). The difference in secretion of RNase I seen between the tissues of these two cultivars may also explain why resistant tissue increased the level of RNase activity in tissue when incubated in buffer alone, while the susceptible cultivar lost activity due to incubation in buffer (Fig 5). An explanation for the differences seen in RNase I activity and in ABA content may be that the resistant cultivar has a greater capacity to convert ABA to phaseic acid. Suggestions for this comes from Harrison and Walton (20), Lin and Ho (28) Pierce and Rasahke (33), and Zeevart (51) who report metabolism of ABA to phaseic acid under stressed conditions.

Tissue from both cultivars, which were desiccated and further incubated in buffer, contained equivalent amounts of protein compared to tissue which was not incubated in buffer (data not shown). These data suggest that the export of RNase from tissue incubated in buffer alone was not due to leakage of cell contents, but due to a differential alteration in the active export programs between the two

cultivars. Since the level of RNase activity increased significantly in 3 hrs in both cultivars due to desiccation, it seems the gain in RNase activity would be to short to be explained by new transcription. These data suggest that increased RNase activity within the tissue and export from the tissue may be due to activation of pre-formed enzyme.

Identification of RNase I Activity from Drought Stressed Tissue: As with results from tissue incubated with 10^{-4} M ABA (Table 2, Fig 3), desiccated tissue from the resistant and susceptible cultivar showed an increase in only RNase I (Fig 8). Likewise, only RNase I was found in the incubation medium from both desiccated resistant and susceptible tissue (data not shown).

Polynucleotide Assays with RNase I: It was of interest to determine if polynucleotide specificity would reveal a particular molecular form of RNase I as being influenced by ABA. RNase I from both cultivars showed no activity towards Poly-G and results for this substrate are not shown. Poly-U showed no difference in RNase specificity between cultivars due to ABA treatment (Table 5). A significant difference was seen in the hydrolysis of Poly-C between cultivars when incubated with RNase I. The resistant cultivar showed greater activity toward Poly-C than did the susceptible cultivar, whether incubated with or without ABA. Increases in RNase I activity due to ABA resulted in a significant increase of activity towards only Poly-A for both cultivars. These results indicate that increases in RNase I due to stimulation by ABA may result from an increase in RNase I specific for Poly-A. Ho and Varner (22) have shown that ABA can

decrease the GA_3-enhanced synthesis of Poly-A-RNA in the barley aleurone layer, suggesting that ABA either increases synthesis or activation of enzymes specific for Poly-A hydrolysis, or inhibits synthesis of new Poly-A-RNA.

RNase Activity after Acclimation: This study involved a continuing effort to describe differences in the mechanisms for processing and distribution of RNase resulting from freeze selection in winter barley. Since desiccation of tissue raised the level of ABA, which appears to alter the secretion and activity of RNase I, it was of interest to determine if cold acclimation might also elevate the level of ABA and in turn might result in detectable alterations to RNase I activity. Several reports (17,25,39) have indicated that treatment with ABA can substitute for cold acclimation. This test was performed to determine if any relationships could be linked between elevated ABA levels and alterations to RNase I activity due to cold acclimation, similar to those seen in desiccated tissue.

The level of ABA in 4-week acclimated tissue from both cultivars was equivalent to that seen in 6-day tissue which was non-acclimated and fully turgid (Fig 6). It is possible that changes in the level of ABA occurred early in the acclimation process and after 4-weeks was again lowered to nonacclimated levels. Table 6 shows that from 0 to 48 hrs after acclimation the level of RNase activity in both cultivars dropped below that of tissue taken directly from acclimation conditions. If ABA did increase early in acclimation, then alterations to RNase may have also occurred prior to the time at which the activity

measurements were conducted. Chen et al. (9) demonstrated that leaves of Solanum commersonii showed a 3-fold increase in free ABA on the 4th day of acclimation and then declined to their initial level. Further studies are needed to fully describe the relationship between ABA and RNase levels in relation to cold acclimation.

SUMMARY

RNase I activity measurements showed that exogenously applied ABA stimulated secretion of the enzyme from leaf tissue of two winter barley cultivars. Whereas enzyme export was about the same for each cultivar, the level of activity in leaf tissue was significantly higher in the freeze-resistant compared to the susceptible cultivar during and following ABA treatment. When the ABA-containing medium was replaced by buffer, the rate of RNase I export was greatly reduced but enzyme activity continued to increase in the resistant cultivar.

Desiccation of leaf tissue for 3 hours caused similar affects on the secretion of RNase I and an increase of activity in the tissue, however, the magnitude of change differed appreciably between the cultivars. Leaf tissue of both cultivars showed equivalent increases in RNase I activity when desiccated. When leaf tissue of both cultivars was incubated in buffer, following desiccation, the susceptible cultivar secreted twice the level of RNase I as compared to desiccated tissue of the resistant cultivar. Increases in RNase I activity caused by desiccation, or due to incubation with exogenous ABA, showed greater specificity for only Poly-A.

Desiccation of leaf tissue caused an accumulation of ABA in both cultivars. The susceptible cultivar showed approximately twice the level of ABA after desiccation compared to the resistant cultivar, which could account for a greater rate of RNase I secretion. Since secretion of proteins has been linked with the endomembrane system

(24), it appears that ABA could be implicated with some alteration to this system. These results suggest evidence for the existance of compartmented, inactive or incomplete forms of RNase I such as those previously reported for other secretory enzymes in yeast mutants (41).

Data reported in this paper indicated that elevated levels of endogeous ABA stimulated differential secretion of RNase I between the two cultivars suggesting that the tissues have different export programs for RNase. Further studies on the alteration and secretion of RNase I due to treatment with biologically active forms of ABA, as well as a study into the changes which occur in ABA and RNase levels during early periods of cold acclimation, may reveal preferential endomembrane pathways or conversions of ABA resulting from freeze selection in winter barley.

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Table 1. Changes in RNase activity from leaf and coleopltile tissue of a freeze-resistant cultivar when floated on ABA solution for varying intervals.

Incubation Interval (hrs)	ABA[a]	Mean (n=2) UA/RA[b]	mg P/ml[c]	Relative Activity
Leaf				
28	-	1.74	1.35	1.28
6	+	2.37	1.25	1.89
12	+	2.94	1.28	2.28
20	+	3.18	1.15	2.75
28	+	3.07	1.16	2.63
36	+	2.81	1.19	2.35
$LSD_{0.05}$		0.33	0.43	0.07
Coleoptile				
28	-	2.98	0.37	7.96
6	+	3.10	0.40	7.64
12	+	3.58	0.44	8.14
20	+	3.29	0.42	7.92
28	+	3.34	0.42	7.91
36	+	2.96	0.41	7.27
$LSD_{0.05}$		NS*	0.07	NS*

[a]Concentration of abscisic acid was 1 x 10^{-4} M.
[b]Units of activity/ RNase assay (See materials and methods).
[c]Mg protein/ml of soluble extraction medium.
*F-Test was not significant ($P < 0.05$).

Table 2. A comparison of Con-A bound and unbound RNase activity from leaf and coleotile tissue of a freeze-resistant cultivar when floated on ABA solution for varying intervals.

Incubation Interval (hrs)	ABA[a]	Mean (n=2) UA/RA[b]	mg P/ml[c]	Relative Activity
Leaf unbound				
28	-	0.91	0.68	1.33
6	+	1.29	0.56	2.30
20	+	1.89	0.47	3.98
36	+	1.52	0.47	3.23
Leaf bound				
28	-	0.17	0.05	3.83
6	+	0.14	0.04	3.42
20	+	0.16	0.04	3.86
36	+	0.21	0.05	4.21
$LSD_{0.05}$		0.12	0.19	0.30
Coleoptile unbound				
28	-	0.37	0.13	2.84
6	+	0.55	0.11	5.03
20	+	0.70	0.09	7.52
36	+	0.69	0.09	7.32
Coleoptile bound				
28	-	0.61	0.04	15.4
6	+	0.74	0.05	16.5
20	+	0.77	0.04	18.7
36	+	0.57	0.04	13.2
$LSD_{0.05}$		0.20	NS*	1.0

[a,b,c] See table 1.

*F-Test was not significant ($P < 0.05$).

Table 3. RNase activity in the bathing media after incubating leaf tissue from a freeze-resistant cultivar for 20 hrs in varying combinations of ABA, DNP, and MES buffer.

Incubation treatment	Mean (n=2)		
	UA/RA[a]	mg P/ml[b]	Relative Activity
20 h MES	0.11	0.04	2.75
6 h ABA, 14 h MES	0.07	0.05	1.40
6 h ABA/DNP, 14 h MES	0.12	0.07	1.71
6 h ABA, 14 h ABA/DNP	0.43	0.07	6.14
20 h ABA	0.25	0.06	4.16
20 h ABA/DNP	0.41	0.07	5.85
$LSD_{0.05}$	0.03	0.08	0.37

[a]Units of activity/ RNase assay.
[b]Mg protein/ ml of soluble extraction medium.

Table 4. RNase activity secreted into the bathing media by leaf tissue from a freeze-resistant compared to a susceptible cultivar when incubated in either ABA, GA_3, or MES buffer. This is a comparison of RNase secretion from leaf tissue which was incubated after a 13% fresh weight lose (desiccated) compared to incubation of non-desiccated leaf tissue.

Incubation Treatment	-13% F.W.	UA/RA[a] Means (n=2) Resistant	Susceptible	Mean[b]
20 h MES	-	0.05	0.08	0.07
20 h ABA	-	0.12	0.15	0.13
14 h MES	+	0.07	0.14	0.11
14 h GA_3	+	0.07	0.10	0.09
Mean[c]		0.08	0.12	
$LSD_{0.05}$		0.01		0.02

[a]Units of activity/ RNase assay (Protein values were below the detectable range and thus regarded as insignificant).
[b]Mean values for each treatment between cultivars.
[c]Mean values between treatments of individual cultivars.

Table 5. A comparison of the hydrolysis rate of polynucleotides by RNase I (without affinity for Con-A) extracted from leaf tissue of a freeze-resistant and susceptible winter barley cultivar. This table compares the hydrolysis rate of RNase I from 6-day non-treated tissue, tissue incubated 20 hrs in MES buffer or ABA, and tissue which was been desiccated followed by 14 hr incubation in MES buffer.

Treatment	-13% F.W.	UA/RA[a] Mean (n=2) Resistant	Susceptible	Mean[b]
		Poly-A		
6-d control	-	0.30	0.23	0.27
20 h MES	-	0.38	0.25	0.32
20 h ABA	-	0.45	0.28	0.37
14 h MES	+	0.48	0.35	0.41
Mean[c]		0.40	0.28	
$LSD_{0.05}$		0.04		0.06
		Poly-C		
6-d Control	-	2.11	0.55	1.33
20 h MES	-	1.20	0.54	0.87
20 h ABA	-	1.66	0.56	1.07
14 h MES	+	1.73	0.42	1.11
Mean[c]		1.67	0.52	
$LSD_{0.05}$		0.04		0.06
$LSD_{0.05}$*		0.09		
		Poly-U		
6-d control	-	1.80	1.75	1.77
20 h MES	-	1.82	1.73	1.77
20 h ABA	-	1.81	1.80	1.80
14 h MES	+	1.85	1.77	1.81
Mean[c]		1.82	1.76	
$LSD_{0.05}$		0.04		0.06

[a]Units of activity/ RNase assay (Protein amount varied between 0.18 and 0.28 mg per assay giving an average of 0.23 mg. This variance was regarded as insignificant in these activity comparisons).
[b]Mean values for each treatment between cultivars.
[c]Mean values between treatments of individual cultivars.
*$LSD_{0.05}$ for the interaction between treatments and tissue.

Table 6. RNase activity (in crude extract) from leaf tissue of a freeze-resistant and susceptible cultivar of winter barley after a 4-week cold acclimation period when incubated in ABA or MES buffer.

Treatment	Incubation[a]	UA/RA[b] Mean (n=2) Resistant	Susceptible	Mean[c]
0 h at 25°C.	-	2.31	2.18	2.25
24 h at 25°C.	-	1.93	1.74	1.84
48 h at 25°C.	-	2.11	1.80	1.96
20 h MES	+	2.31	1.62	1.97
20 h ABA	+	2.63	1.47	2.05
Mean[d]		2.25	1.76	
$LSD_{0.05}$		0.07		0.10
$LSD_{0.05}$*		0.15		

[a]Incubation of excised, 4-week acclimated leaf tissue = +.
[b]Units of activity/ RNase assay (Protein amount varied between 0.30 and 0.46 mg per assay giving an average of 0.40 mg. This variance was regarded as insignificant in these activity comparisons).
[c]Mean values for each treatment between cultivars.
[d]Mean values between treatments of individual cultivars.
*$LSD_{0.05}$ for interaction between treatments and tissue.

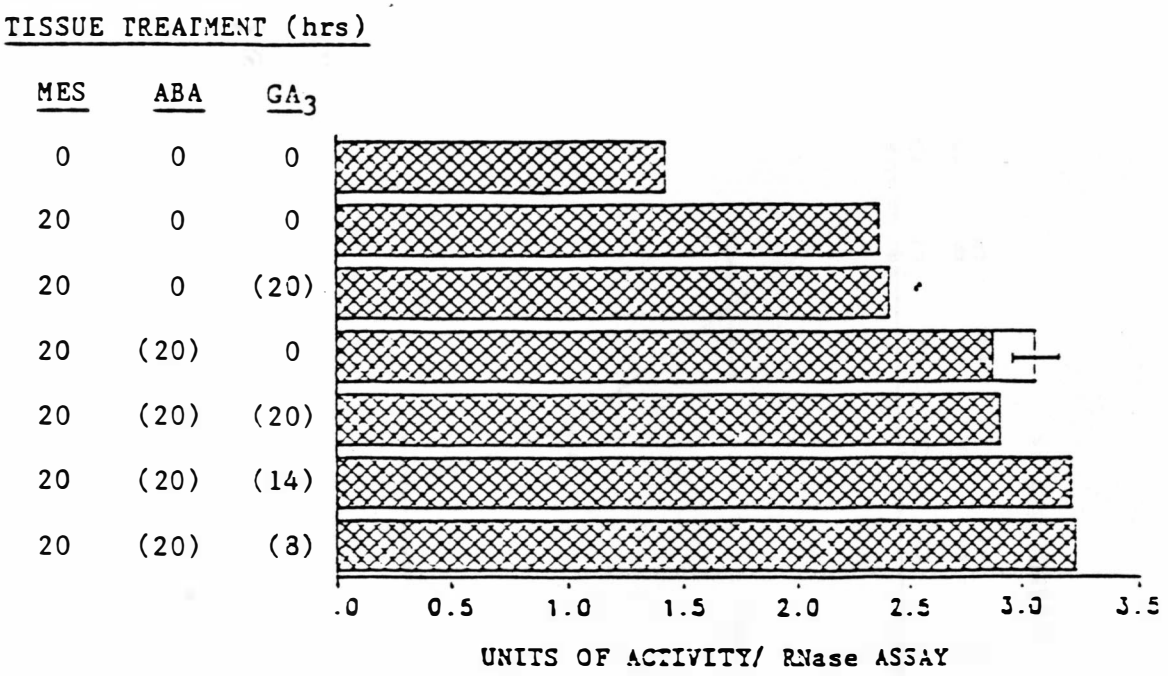

Figure 1. Changes in units activity/ RNase assay from freeze-resistant leaf tissue which was incubated for varying intervals with 20 mM MES buffer, 10^{-4} M ABA, or 10^{-4} M GA_3. Protein amount varied between 0.20 and 0.26 mg per assay giving an average of 0.22 mg. This variance was regarded as insignificant in these activity comparisons. Values in parenthesis indicate length of time that tissue was exposed to each of the amendments, and where lesser values indicate addition at the beginning of the incubation intervals. Combined total incubation of tissue never exceeded 20 hrs. Single bar line = $LSD_{0.05}$.

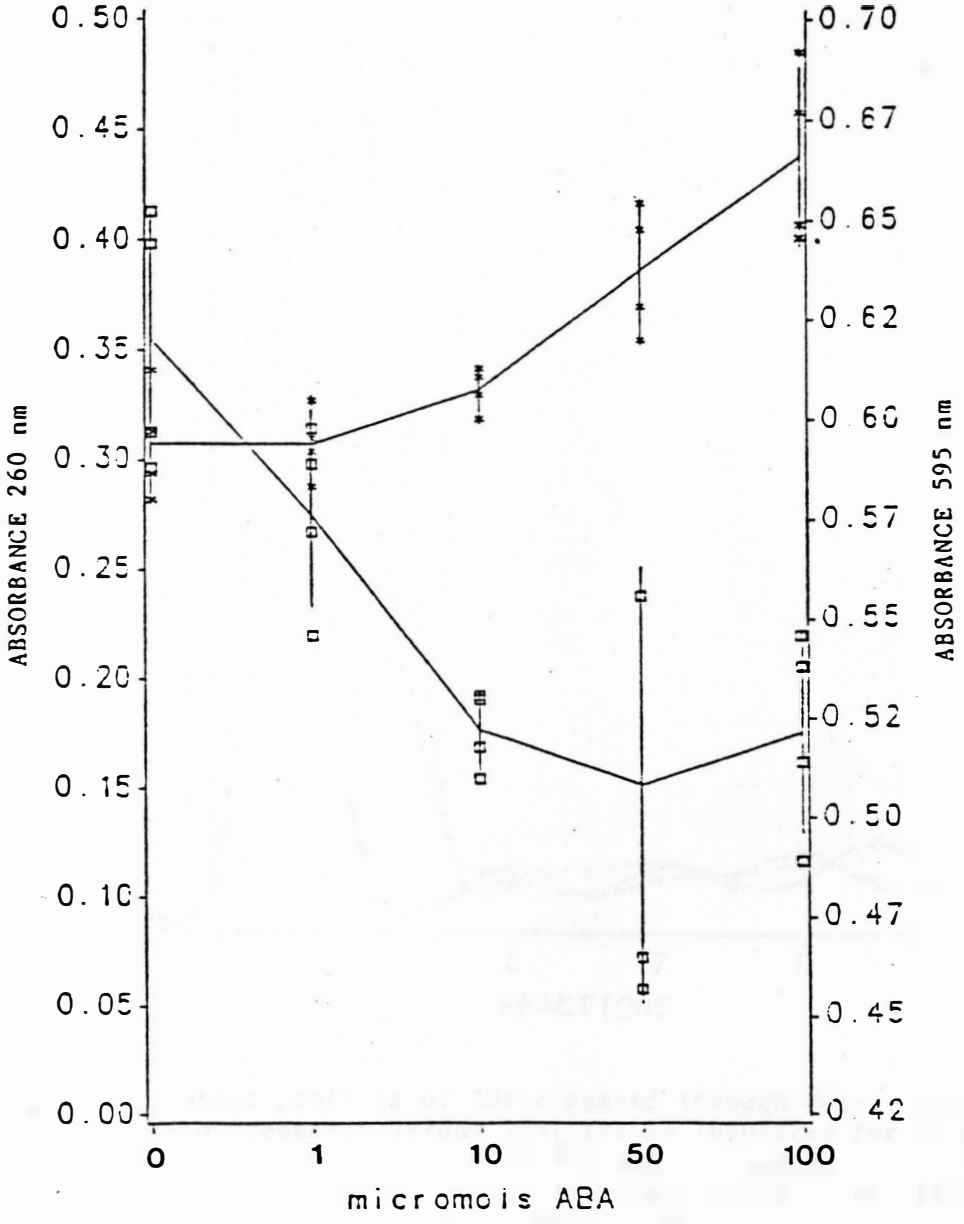

Figure 2. Changes in RNase activity (x) and soluble protein (□) from freeze-resistant leaf tissue incubated in ABA ranging from 0 to 100 micromolar. RNase activity was measured at A_{260} and protein was measured at A_{595}.

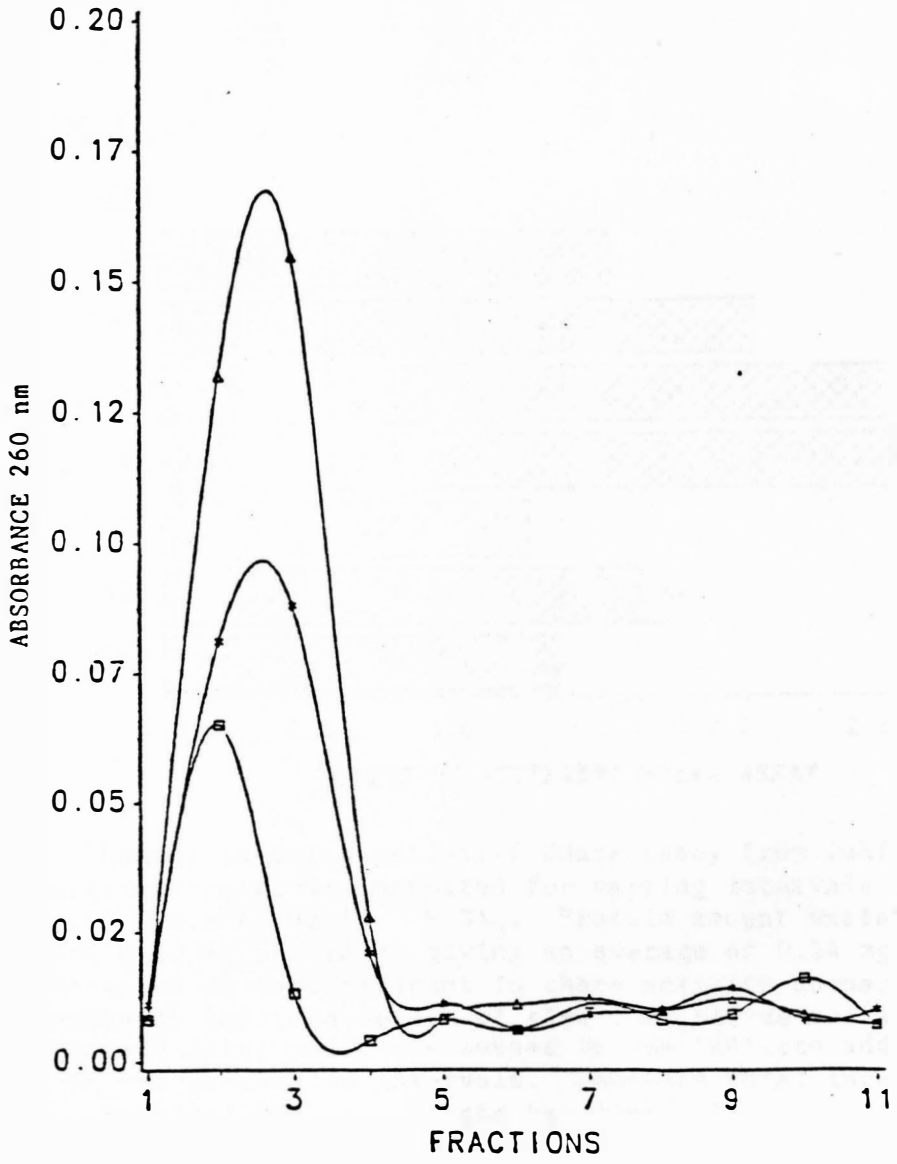

Figure 3. Elution profiles of RNase passed through Con-A, which were secreted from freeze-resistant leaf tissue incubated for either 28 hr in 20 mM MES buffer (□), 6 hr in 10^{-4} M ABA (×), or 20 hr in 10^{-4} ABA (△). Each fraction (1 to 6), which contained RNase I, was eluted with 2 ml of 50 mM Tris/HCl (pH 7.4), containing 0.1 mM each $CaCl_2$, $MgCl_2$, $MgCl_2$ and 0.01% Triton X-100. Fractions 7 to 10, which contained RNase II, were eluted with the same buffer, which contained 500 mM alpha-methyl-D-mannopyranoside. RNase activity was measured at A_{260}

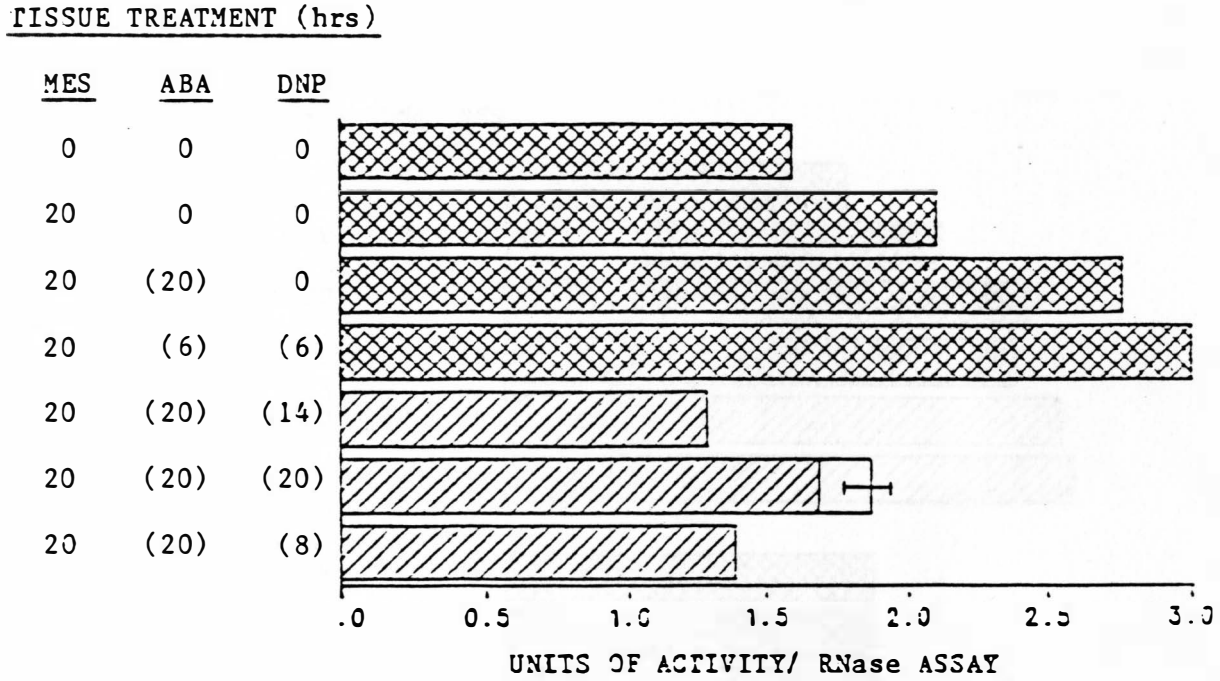

Figure 4. Changes in units activity/ RNase assay from leaf tissue of a freeze-resistant cultivar incubated for varying intervals with 20 mM MES buffer, 10^{-4} M ABA, or 10^{-4} M GA_3. Protein amount varied between 0.09 and 0.20 mg per assay giving an average of 0.14 mg. This variance was regarded as insignificant in these activity comparisons. Values in parenthesis indicate length of time that tissue was exposed to each of the amendments, and where lesser values indicate addition at the beginning of the incubation intervals. Combined total incubation of tissue never exceeded 20 hrs. Single bar line = $LSD_{0.05}$.

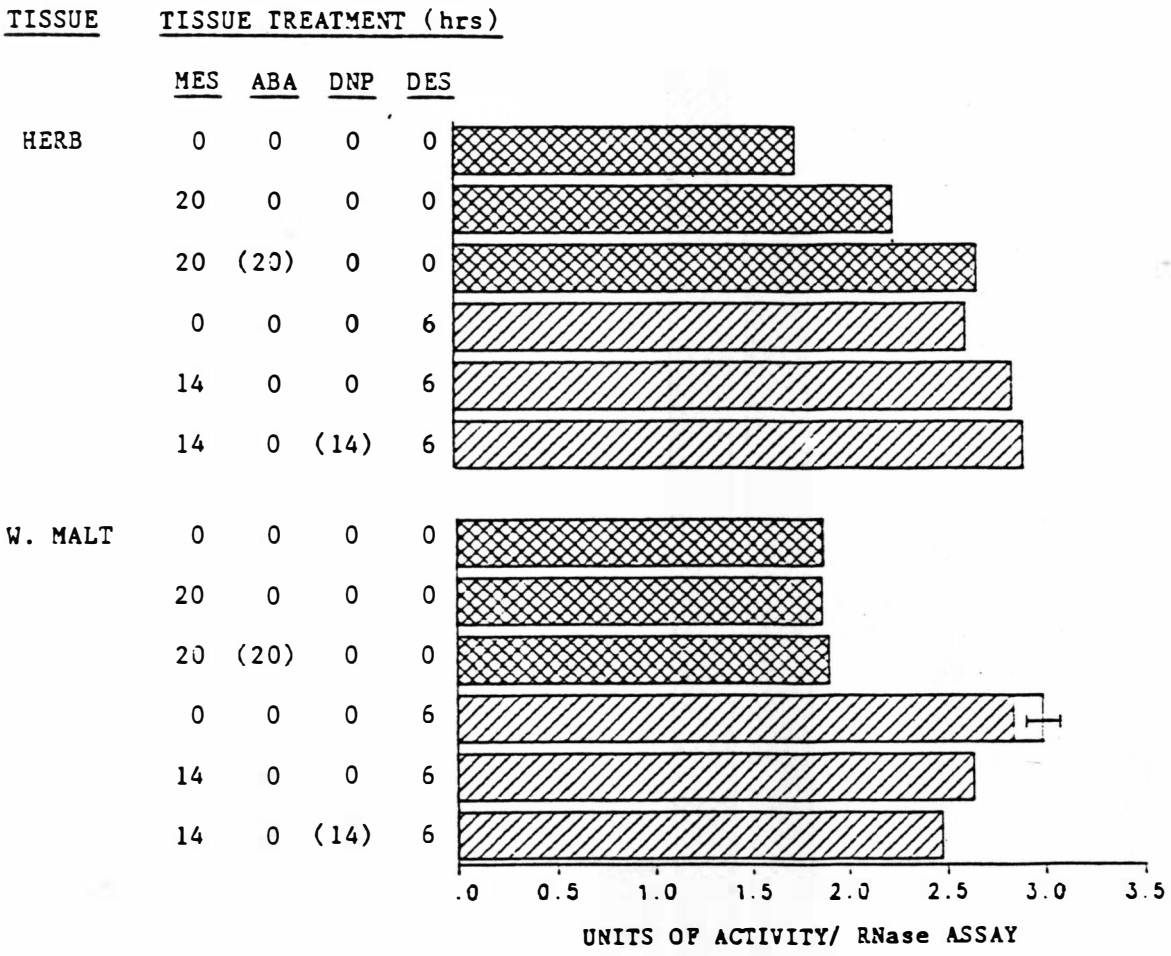

Figure 5. A comparison of units activity/ RNase assay containing extracts from 6-hr desiccated or non-desiccated leaf tissue each of a freeze-resistant (Herb) and susceptible (W. Malt) cultivar incubated previously in either 20 mM MES buffer, 10^{-4} M ABA, or 10^{-4} M GA_3. Protein amount varied between 0.19 and 0.24 mg per assay giving an average of 0.22 mg. This variance was regarded as insignificant in these activity comparisons. Values in parenthesis indicate length of time that tissue was exposed to each of the amendments. Combined total incubation of tissue never exceeded 20 hrs. Single bar line = $LSD_{0.05}$.

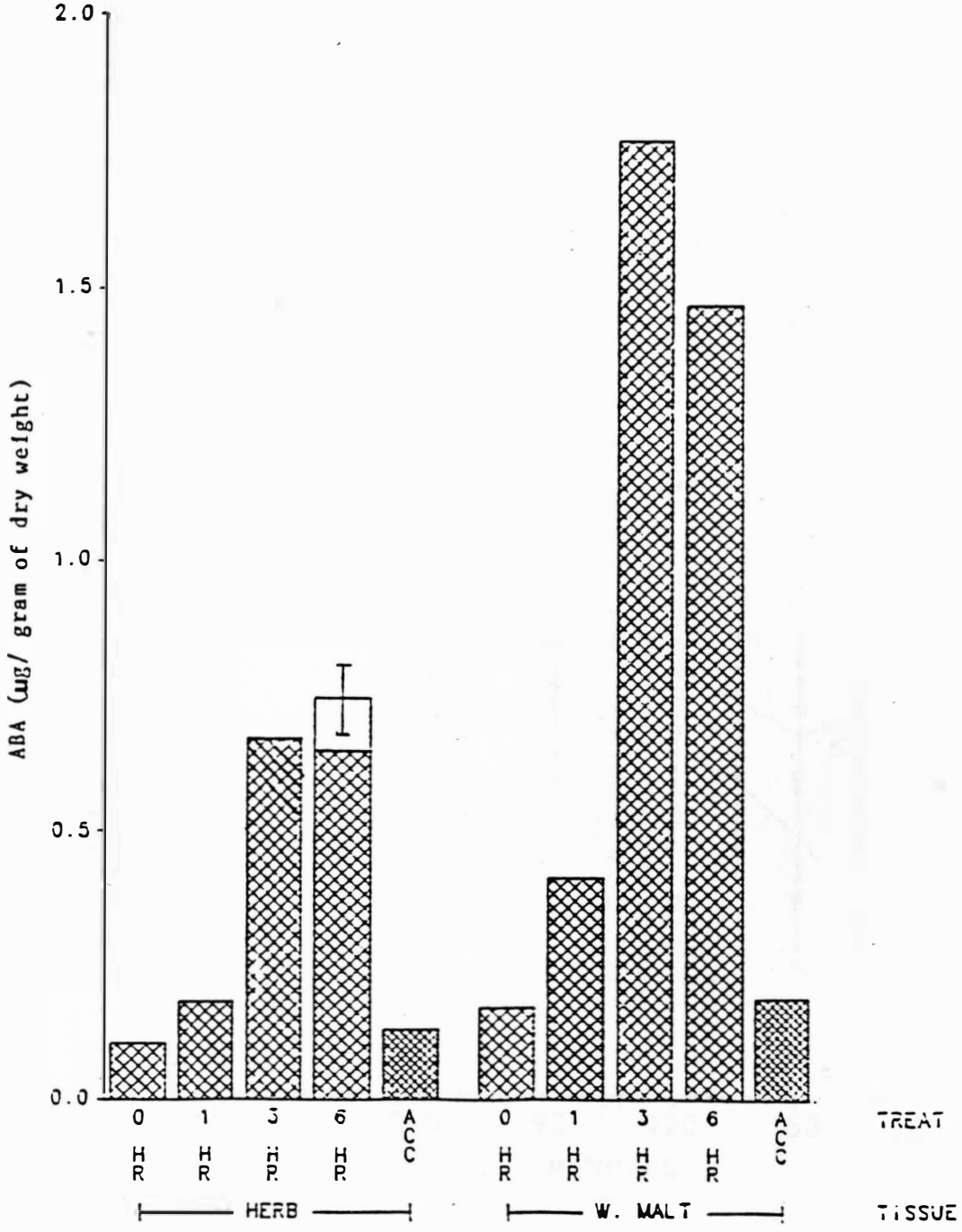

Figure 6. Leaf tissue accumulation of ABA during a 6 hr-desiccation period compared to 4-week cold acclimated tissue from a freeze-resistant and susceptible cultivar of winter barley. ABA samples were derivatized with diazomethane and quantitated on a Varian 3700 GLC equipped with a [Ni^{63}] ECD. Single bar line = $LSD_{0.05}$

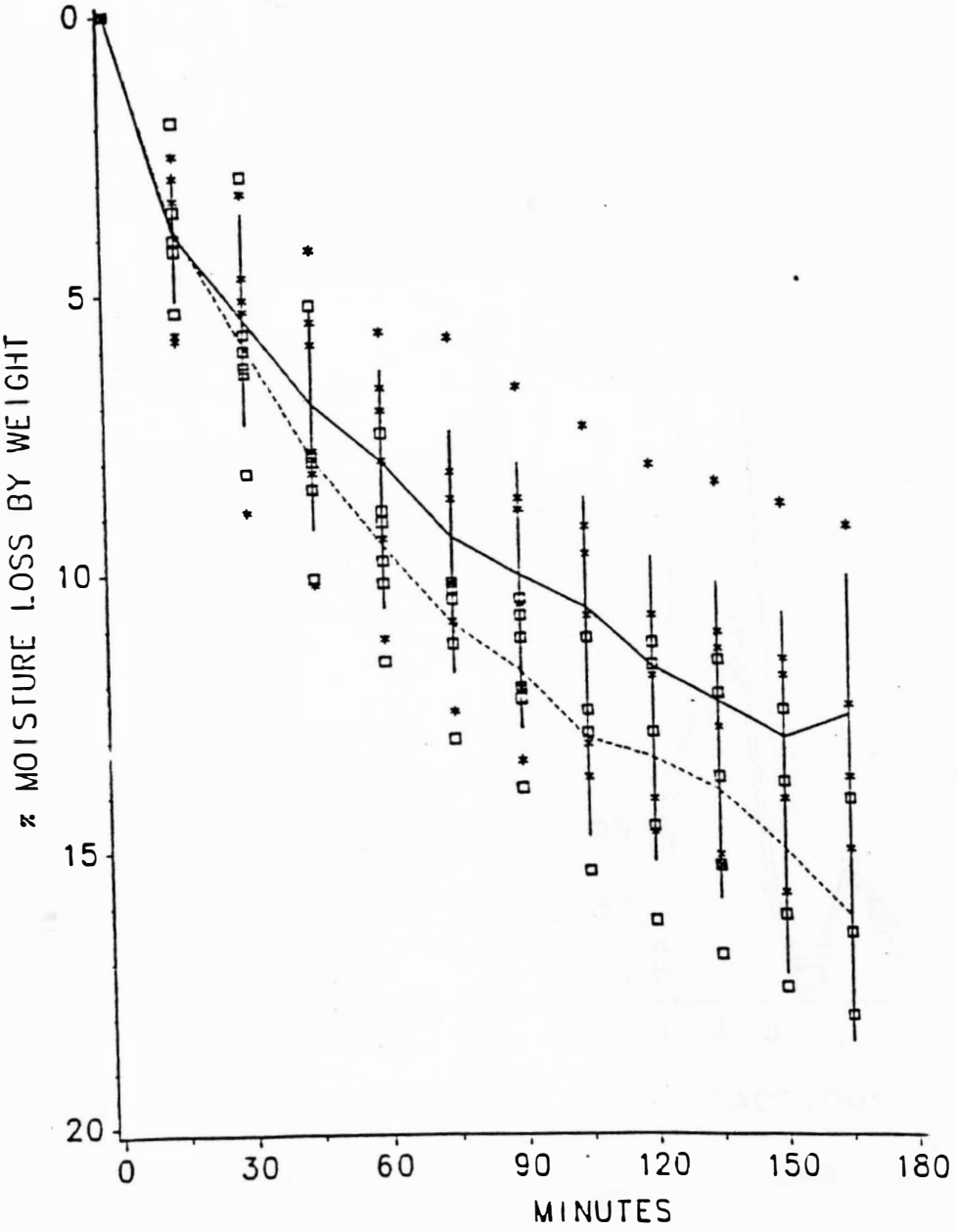

Figure 7. A comparison of the average % water loss from excised leaf tissue of both a freeze-resistant (——) and susceptible (----) cultivar of winter barley. (x) indicates data points for the resistant cultivar and (□) indicates data points for the susceptible cultivar.

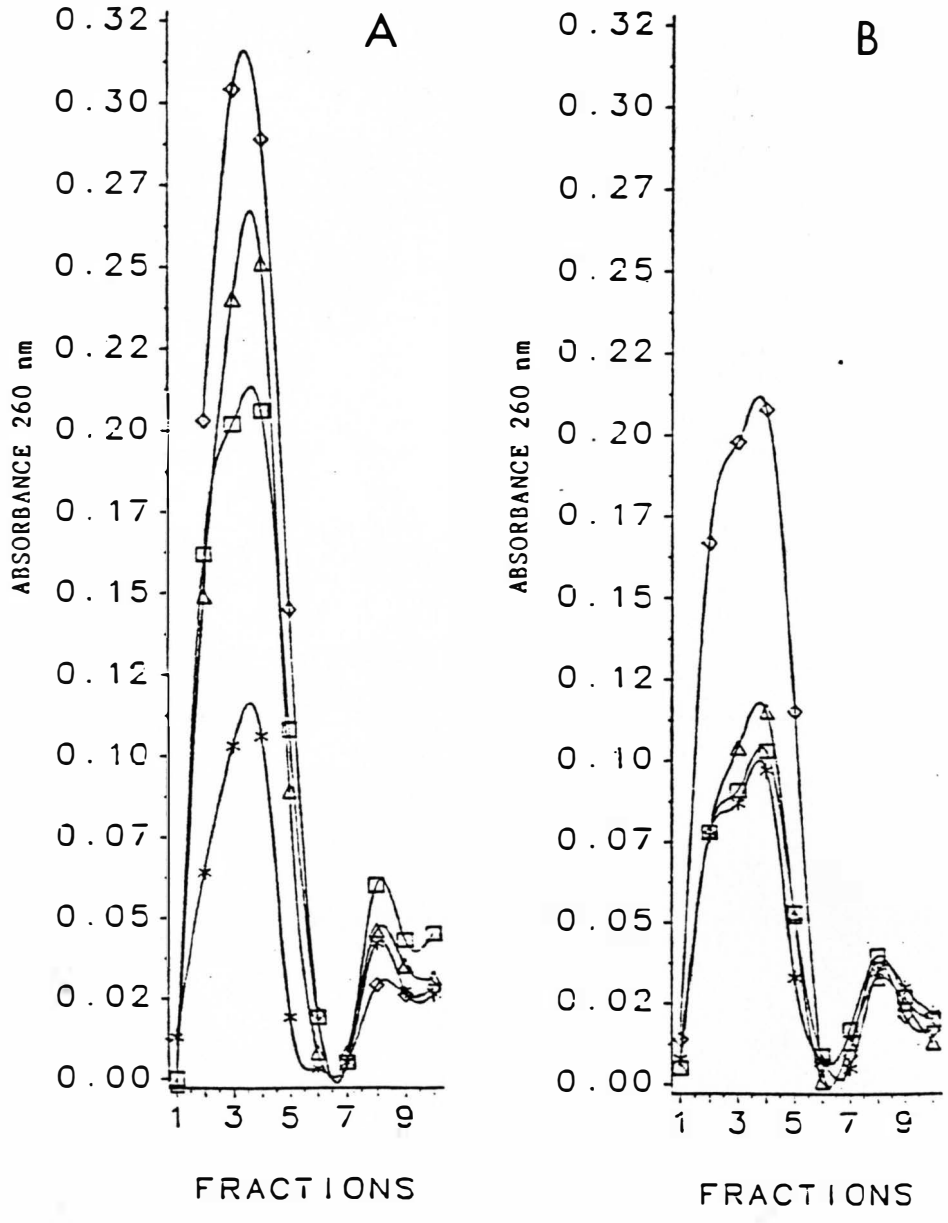

Figure 8. Elution profiles of RNase activity partitioned from crude extracts between Con-A bound and unbound fractions from both a freeze-resistant [A] and susceptible [B] cultivar of winter barley. Extracts were obtained from 2-gram samples of leaf tissue which recieved the following treatments: (x) 6 day control, (□) 20 hr incubation in 20 mM MES buffer, (△) 20 hr incubation in 10^{-4} M ABA, and (◇) 6 hr desiccation, followed by 14 hr in 20 mM MES buffer. Each fraction (1 to 6), which contained RNase I, was eluted with 2 ml of 50 mM Tris/HCl (pH 7.4), containing 0.1 mM $CaCl_2$, $MgCl_2$, $MnCl_2$ and 0.01% Triton X-100. Fractions 7 to 10, which contained RNase II, were eluted with the same buffer, which contained 500 mM alpha-methyl-D-mannopyranoside. RNase activity was measured at A_{260}.